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(Article begins on next page)

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MELATONIN PHARMACOKINETICS AND DOSE EXTRAPOLATION AFTER ENTERAL INFUSION IN NEONATES SUBJECTED TO HYPOTHERMIA

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Running Title: Melatonin pharmacokinetics in hypothermia

Abstract

Introduction: Neonates with hypoxic-ischemic encephalopathy (HIE) undergoing hypothermia may benefit from adjunctive therapy with melatonin. However, melatonin safety, pharmacokinetics (PK), and dosage in this sensitive population is still unknown.

Methods and results: This study assessed the PK and safety of melatonin enteral administration to neonates with HIE undergoing hypothermia. Melatonin was infused at 0.5 mg/kg in five neonates with HIE undergoing hypothermia. Infusion started 1 h after the neonates reached the target temperature of 33.5 °C. Blood samples were collected before and at selective times after melatonin infusion. Abdominal complications or clinically significant changes in patients' vital signs were not found during or after melatonin. The peak plasma concentration reached 0.25 µg/ml. The area under the curve in 24 h was 4.35 µg/mL*h.

Discussion: Melatonin half-life and clearance were prolonged, and the distribution volume decreased compared to adults. *In silico* simulation estimated that the steady state can be reached after four infusions. Hypothermia does not affect melatonin PK. In humans high blood concentrations with lower doses can be achieved compared to animal experimentation, although intravenous administration is advised in the neonate population. Our study is a preparatory step for future clinical studies aimed at assessing melatonin efficacy in HIE.

KEYWORDS

melatonin, pharmacokinetics, hypothermia, infants, immaturity, neuroprotection, hypoxic-ischemic encephalopathy

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1 | INTRODUCTION

Hypoxic-ischemic encephalopathy (HIE) is the major cause of neurological morbidity in the term infant. Currently, therapeutic hypothermia is the only approved medical treatment to minimize brain injury in infants with HIE. Research has shown that therapeutic hypothermia is beneficial in term and late preterm neonates with HIE. To be effective HIE must be applied within six hours of the baby's birth.¹ However, therapeutic hypothermia only provides beneficial outcomes in less than 50% of the treated patients.¹⁻³ Therefore, effective pharmacological therapeutic agents able to be used as an adjunct to hypothermia are needed to improve neonatal outcome.

Preclinical studies have shown that melatonin improves hypothermia efficacy.⁴⁻⁶

Melatonin is a naturally occurring substance mainly produced from the pineal gland and well known for its role in regulating the circadian rhythm. However, Melatonin also possesses pleiotropic effects including free radical scavenger activity and stimulation of anti-oxidant enzymes. In addition, melatonin exhibits immunomodulatory effects in the presence of an exacerbated immune response.⁷⁻⁹ Due to these pleiotropic and immunomodulatory effects, melatonin can reduce several key components involved in the development of hypoxic-ischemic injury. Studies in preclinical models of perinatal HIE have demonstrated that melatonin minimizes cell death and improves outcomes.¹⁰⁻¹⁴ Human studies have also provided evidence of efficacy. In asphyxiated neonates, melatonin administered at the dose of 10 mg PO every 2 hours for 8 doses reduced malondialdehyde and nitrate/nitrite concentrations without side effects when compared to the infants given placebo. This reduction in malondialdehyde and nitrate/nitrite concentrations indicate that lipid peroxidation and oxidative damage were reduced.¹⁵ A recent study examined the effect of five daily enteral doses of melatonin on clinical, biochemical, neurophysiological, and radiological outcomes of neonates with HIE. Melatonin-treated neonates in this study had decreased brain injury compared to controls.¹⁶

The evidence from preclinical studies indicates that melatonin is a remarkable neuroprotective therapy, and melatonin's use may help avoid the sequelae of HIE in neonates.¹⁷ However, the safety, pharmacokinetics (PK), dosing, and effectiveness of melatonin in infants with HIE undergoing hypothermia is still unclear. Thus, trials aimed at assessing safety and PK must occur prior to large randomized controlled trials. To protect the vulnerable neonatal population, all information collected during a drug trial must be carefully evaluated. In addition, PK data must be evaluated to find an appropriate dosing schedule. The

PK data collected during these studies will provide the first step in describing the dose- concentration and dose- toxicity response relationships, which is essential in designing a future clinical trial protocol.

Here we report the first results of a clinical study approved by the FDA (ClinicalTrials.gov Identifier: NCT02621944). The aim of this study was to establish the safety and PK of melatonin after enteral administration to neonates with HIE undergoing hypothermia. In this study we used a novel manufactured preparation of melatonin (Int. Pat. Appl. PTC/IB2016/051235) that did not contain excipients like alcohol or other substances used to increase melatonin solubility. The absence of excipients helped reduce the possibility of side effects from exogenous substances.

2| Materials and Methods

2.1 | Study population

The study was designed and conducted in accordance with the Declaration of Helsinki and approved by The University of Florida Institutional Review Board (ClinicalTrials.gov Identifier: NCT02621944). Prior to enrolment in the study, written informed consent was obtained from the parents of five neonates admitted to the Neonatal Intensive Care Unit at the University of Florida, Gainesville, USA. Subjects were enrolled in the study within 24 hours of birth. Patients with HIE eligible for hypothermia therapy were recruited. Entry criteria for hypothermia included a gestational age of 36 weeks or greater, a birth weight of 1.8 kg or greater, and less than or equal to 6 hours of age. The neonates had evidence of moderate or severe encephalopathy as defined by seizures or abnormalities on a modified Sarnat exam (level of consciousness, spontaneous activity, posture, tone, primitive reflexes including suck and Moro, and autonomic system findings including pupils, heart rate, and respirations).

Evidence of hypoxic-ischemic injury was defined as 1) a pH of 7.0 or less and/or a base deficit of greater than 16, or 2) a pH between 7.01-7.15 and/or a base deficit between 10-15.9, or 3) no blood gas available and an acute perinatal event (cord prolapse, fetal bradycardia, uterine rupture).¹⁸ Neonates with suspected inborn errors of metabolism (elevated ammonia) and hypoglycemia, clinical signs and symptoms consistent with meningitis detected upon sepsis evaluation, or a diagnosis of congenital abdominal surgical problems along with multiple congenital anomalies and/or chromosomal abnormalities were excluded.

2.2 | Dose and medication

A stable and sterile aqueous melatonin solution (0.4 mg/ml) free from excipients (Int. Pat. Appl. PTC/IB2016/051235) was prepared by Industria Farmaceutica Galenica Senese S.R.L. (Monteroni d'Arbia, Siena, IT). Neonates enrolled in the study received the first dose of melatonin within 24 hours of life with a target of 12 hours of life or less. The drug was infused via enteral route through an OG tube at a dose of 0.5 mg/kg. The infusion started 1 hour after the neonates reached the target temperature of 33.5°C (hypothermia). Prior to administration, the investigational drug pharmacy at the University of Florida diluted the melatonin with normal saline to a final concentration of 6 ml. A feeding pump infused the solution over 4 hours at a rate of 1.5 ml/h. The standardized volume, infusion time, and dose allowed for comparisons of the pharmacokinetics between subjects.

Blood samples (1 ml) were collected from an indwelling arterial catheter immediately before (time 0) and 1, 3, 6, 12, 24, 48, and 96 hours after starting the melatonin infusion. Blood samples were collected in blood collection tubes containing lithium heparin (cat #367884, BD Vacutainer®, Franklin Lakes, NJ) and stored at 4°C until centrifugation. The

sample was centrifuged for 10 minutes at 2000g at 4°C. The plasma (500 µL) was transferred to a 2.0 ml red cap self-standing microcentrifuge tube (cat # 1420-9704, USA Scientific, Ocala, FL) and immediately stored at - 80°C.

2.3 | Melatonin tolerance

During the entire period of melatonin administration, the neonate was monitored using vital monitoring (heart rate, respirations, blood pressure), systemic and cerebral saturations, and abdominal exams. Specifically, the Vital Sync™ (Medtronic, formerly Covidien, Minneapolis, MN) monitoring platform captured vital sign data. Every 30 seconds, the Vital Sync™ captured the cerebral saturation plus the heart rate, arterial blood pressure, cuff blood pressure, and pulse oximeter saturation of each patient. INVOS™ Cerebral/Somatic Oximetry (Medtronic, formerly Covidien, Minneapolis, MN) captured cerebral saturations. Infant-Neonatal Sensors with near-infrared spectroscopy measured regional mixed venous-arterial saturations. Sensors were placed over either the left or right side of the forehead. Data were captured prior to, during, and after melatonin infusion. The data were exported from the Vital Sync™ as a spreadsheet and prepared for analysis. Abdominal exams that included abdominal circumference were performed prior to and hourly during the four-hour infusion.

2.4 | Melatonin concentration assay

Plasma melatonin concentrations were measured by high-performance liquid chromatography (HPLC) and mass spectrometry (MS/MS) (Agilent Technologies 1200 series system and an ABSciex API 4000 triple-quadrupole mass spectrometer) according to the method of Wang et al.,¹⁹ as previously described.²⁰

2.5 | Pharmacokinetic modeling and analysis

Individual patient data and population data were analyzed by adapting the classical one-compartment model for intravenous (IV) infusion to nasogastric infusion. The classical one-compartment model for IV infusion is based on a constant rate of infusion. The model divides the function of concentration (C) vs time (t) into two different intervals:

$$1) \ C(t) = \begin{cases} C(t) = A(1 - \exp(-K_{el}t)) & t \leq t_{inf} \\ C(t) = A(\exp(-K_{el}(t - t_{inf})) - \exp(-K_{el}t)) & t > t_{inf} \end{cases}$$

where t_{inf} is the time of IV infusion, A is a constant, and K_{el} is the elimination constant. In the case of nasogastric infusion, however, the model leads to an incorrect fitting due to the time-delay caused by absorption through the gastric mucosa. To overcome this problem, a term was introduced that took into account this delay. This term, defined as t_{abs} , considers that the absorption rate is constant. The new equation is thus:

$$2) \ C(t) = \begin{cases} C(t) = A(1 - \exp(-K_{el}t)) & t \leq t_{inf} + t_{abs} \\ C(t) = A(\exp(-K_{el}(t - t_{inf} - t_{abs})) - \exp(-K_{el}t)) & t > t_{inf} + t_{abs} \end{cases}$$

Because t_{abs} is not directly measurable, we estimated t_{abs} by optimizing its value through maximization of R^2 . Using the PK Solver[®] 2.0 software,²¹ we computed R^2 for virtual values of t_{inf} ranging from 4 to 10 h with steps of 0.1 h in order. This procedure enabled us to detect the t_{inf} value that maximized R^2 . The best estimates of t_{abs} were found through the difference obtained between the virtual value of t_{inf} and its real value, i.e. $t_{inf} = 4$ h. For the

measured data set, we obtained an estimated t_{abs} of 2.8 h with a virtual value of $t_{inf} = 6.8$ h.

These values corresponded to $R^2 = 0.995$, indicating an excellent goodness of fit.

The peak concentration time (t_{max}) and the maximal concentration (C_{max}) were then calculated as follows:

$$3) \quad t_{max} = t_{inf} + t_{abs}$$

$$4) \quad C_{max} = A \left(1 - \exp \left(-K_{el}(t_{inf} + t_{abs}) \right) \right)$$

2.6 | Simulation of plasma concentration time profiles following repeated melatonin infusions.

We used the real data obtained in the neonatal PK study to estimate *in silico* the PK profile of repeated melatonin infusions. The simulation was based on the hypothesis that after each administration, melatonin followed the same PK of the first infusion. For a generic k^{th} infusion, the model can be described as:

$$5) \quad \sum_{i=1}^k C(t - 24(i - 1))$$

where time (t) is in hours. Using this model, we simulated the administration of seven infusions repeated every 24 h.

3 | RESULTS

3.1 / *Demographics*

The average gestational age of the enrolled subjects was 39.8 ± 0.75 weeks with an average birthweight of 3.46 ± 0.05 kg. Of the five subjects, four were male, and one was female. The remainder of the demographics are summarized in Table 1. Below is a short description of the clinical course of each subject.

3.2 / *Clinical Data and Tolerance to melatonin*

Patient M001 was outborn at an estimated gestational age (EGA) of 39 weeks. Delivery occurred by C-Section due to non-reassuring fetal heart tones during labor. At delivery, a placental abruption was noted. Apgar scores were 1/1/1/3 at 1', 5', 10', and 15', respectively. The patient required cardiopulmonary resuscitation (CPR) for 3 minutes and was intubated at 5 minutes of life. The cord blood gas demonstrated pH 6.895, PaCO₂ 73 mm Hg, PaO₂ <16 mm Hg, HCO₃ 14 mEq/L, and a base excess (BE) -19 with a lactic acid of 16.6mmol/L. The initial Sarnat score was stage II. The neonate was intubated for 2 days, including during the melatonin infusion. The melatonin infusion was started at 8 hours of life. The HR gradually decreased from a mean of 117 ± 7 bpm prior to infusion to 109 ± 7 bpm during the infusion and 95 ± 7 bpm for 12 hours after the infusion (Figure 1A, panel *a*). Cerebral and systemic saturations and the MAP remained constant prior to, during, and after the infusion (Figure 1A, panels *b-d*). The baseline abdominal circumference was 31 cm and increased to 32 cm by the completion of the infusion (Figure 2). The initial abdominal exam revealed a full but soft abdomen that was not tender to palpation with active bowel sounds. The final exam revealed a full but soft abdomen that was not tender to palpation. An MRI obtained at day of life (DOL) 4 did not have evidence of hypoxic-ischemic injury. Small foci

of hemorrhage were present in both cerebellar hemispheres. The MRI also showed a small-volume subdural hematoma posteriorly on the right. The subject was discharged on DOL 11.

Patient M002 was inborn at the EGA of 41 weeks. Delivery occurred via emergent C-section due to non-reassuring fetal heart tones. Apgar scores were 1/2/3 at 1', 5', and 10', respectively. The arterial cord gas results were as follows: pH 7.24, PaCO₂ 61 mmHg, PaO₂ 14.3 mmHg, HCO₃ 14 mEq/l, and BE -3.8. The neonate's initial arterial gas results were pH 7.21, PaCO₂ 33 mmHg, PaO₂ 147 mmHg, and BE -15. The neonate's initial Sarnat score was stage III. The patient was intubated for 4 hours and then extubated to room air. The neonate was on room air during the melatonin infusion. The infusion was started at 14 hours of life. The HR mean was 84±19 bpm prior to infusion, 70±10 bpm during the infusion, and 107±17 bpm after the infusion (Figure 1B, panel *e*). The MAP was 76±3 mmHg prior to infusion, 73±6 mmHg during the infusion, and 68±5 mmHg after the infusion (Figure 1B, panel *f*). The systemic and cerebral saturations were unchanged prior to, during, and after the infusion (Figure 1B, panels *g-h*). The abdominal circumference was 31.5 cm prior to the infusion and decreased to 31.0 cm by the completion of the infusion (Figure 2). The abdominal exam at baseline revealed a non-tender, non-distended abdomen with active bowel sounds. The exam was unchanged at the completion of the infusion. The MRI obtained after the completion of hypothermia on DOL 3 did not reveal any hypoxic-ischemic injury. The patient was discharged on DOL 7.

Patient M003 was inborn at the EGA of 40 weeks. The delivery was complicated by a prolonged rupture of membranes, chorioamnionitis, shoulder dystocia, and a nuchal cord. After delivery, the patient did not have any respiratory effort and had a heart rate less than 60 bpm, poor tone, and poor color. The patient's airway was suctioned with a catheter. Then, the patient was placed on positive pressure ventilation with 100% oxygen. The heart rate improved with positive pressure ventilation. Apgar scores were 1/6 at 1' and 5', respectively.

The arterial cord gas results were as follows: pH 7.06, PaCO₂ 79 mmHg, PaO₂ 23 mmHg, HCO₃ 21 mEq/l, and BE -12.3. The arterial blood gas results on admission were as follows: pH 7.38, PaCO₂ 19 mmHg, PaO₂ 99 mmHg, HCO₃ 18 mEq/l, and BE -7.0. The initial Sarnat score was stage II. The patient was placed on continuous positive airway pressure for 24 hours. The melatonin infusion was started at 6 hours of life and occurred during the time of continuous positive airway pressure. The HR mean was 108±13 bpm prior to infusion, 84±13 bpm during the infusion, and 90±20 bpm after the infusion (Figure 1C, panel *i*). The mean cerebral oximetry was 81±2% prior to infusion, 68±8% during the infusion, and 75±3% after the infusion (Figure 1C, panel *n*). The systemic saturations and MAP were unchanged prior to, during, and after the infusion (Figure 1C, panels *l* and *m*). The abdominal circumference was 34.5 cm prior to the infusion and remained unchanged at the completion of the infusion. The abdominal exam at baseline revealed a non-tender, non-distended abdomen with active bowel sounds. The abdominal exam was unchanged at the completion of the infusion (Figure 2). The MRI at DOL 5 demonstrated no evidence of hypoxic-ischemic injury. The subject was discharged on DOL 7.

Patient M004 was outborn at the EGA of 40 weeks. Delivery occurred via spontaneous vaginal delivery, and the neonate was limp and apneic at delivery. The health care team found the neonate difficult to resuscitate via bag valve mask, so the neonate was intubated. The neonate continued to require high respiratory pressure support. The heart rate diminished, and CPR was initiated. In addition, the patient received several rounds of epinephrine for a low heart rate. The subject had bilateral tension pneumothoraces. Bilateral chest tubes were placed, and the heart rate improved. The initial cord blood gas was as follows: pH 6.99, PaCO₂ 25 mmHg, PaO₂ 98 mmHg, HCO₃ 23 mEq/l, and BE -9.0. The initial Sarnat score was stage II. The patient was given one dose of surfactant and required high frequency oscillator ventilation and inhaled nitric oxide due to persistent pulmonary

hypertension of the newborn (PPHN) and meconium aspiration syndrome (MAS). The melatonin infusion was started at 9 hours of life. The cerebral oximetry mean was $71\pm 9\%$ prior to infusion, $77\pm 7\%$ during the infusion, and $85\pm 7\%$ after the infusion (Figure 1D, panel *r*). The neonate's heart rate, systemic saturations, and MAP were unchanged prior to, during, and after the infusion (Figure 1D, panels *o-q*). The abdominal circumference was 32 cm prior to the infusion and was unchanged at the completion of the infusion (Figure 2). The abdominal exam at baseline revealed a full but soft abdomen without active bowel sounds. The exam was unchanged at the completion of the infusion. The MRI obtained on DOL 8 demonstrated a subdural haemorrhage and a 1.2 cm parenchymal hemorrhage in the left occipital lobe, but no evidence of hypoxic-ischemic injury. The subject was discharged on DOL 21.

Patient M005 was outborn at an EGA of 39 weeks. Delivery occurred via emergent C-section due to non-reassuring fetal heart tones. Meconium stained fluid was noted at delivery. The neonate's Apgars were 1/5/7 at 1', 5', and 10', respectively, and the subject required intubation at delivery. An initial venous blood gas upon arrival to the NICU was as follows: pH 7.03, PaCO₂ 56 mmHg, PaO₂ 68 mmHg, HCO₃ 15 mEq/l, BE -17. The neonate's initial Sarnat score was stage II. The neonate required high frequency oscillator ventilation and inhaled nitric oxide and was evaluated for possible extracorporeal membrane oxygenation (ECMO). The melatonin infusion was started at 9 hours of life. The heart rate, systemic saturations, MAP, and cerebral saturations were unchanged prior to, during, and after the infusion (Figure 1E). The abdominal circumference was 31 cm prior to the infusion and 31.5 cm at the completion of the infusion (Figure 2). The abdominal exam at baseline revealed a non-tender, non-distended abdomen without active bowel sounds. The exam done at the completion of the infusion revealed a soft abdomen with active bowel sounds. An MRI on

DOL 9 revealed a small punctate focus of T1 signal in the right periventricular white matter. The subject was discharged at DOL 30.

3.3 / *Melatonin pharmacokinetics*

We applied the modified one-compartment model for intravenous infusion to the plasma concentrations of melatonin detected after 0.5 mg/kg intragastric infusion. The dose, calculated for the body weight of each neonate, was administered over 4 hours. The data of the individual patients and the population were analyzed at the selected times before and after the end of the infusion.

The measured concentrations of melatonin in plasma are reported in Table 2. Of the 5 neonates studied, 4 showed median basal serum melatonin concentrations of 20.6 pg/mL. A baseline melatonin concentration was not detected in patient M003. The highest plasma melatonin concentrations were obtained between 3 and 12 h after the end of the infusion (time points 7 and 16 h, respectively). The measured peak concentration varied greatly among patients (0.08-0.28 µg/mL).

Table 3 reports the estimated PK parameters in individual patients as mean±SE. As shown, the individual mean maximal serum concentration (C_{max}) was 0.27 ± 0.04 µg/mL (0.25 µg/mL in the population analysis), whereas the individual mean time to maximal concentration (T_{max}) was 8.66 ± 1.12 h (6.8 h in the population analysis). Using the modified one compartment model analysis, the individual patients had a an elimination half-life ($T_{1/2}$) of 50.90 ± 36.43 h (26.43 h in the population analysis), a volume of distribution (V) of 5.67 ± 0.08 L, and a clearance (CL) of 0.21 ± 0.07 L/h. The V and CL data obtained in the population analysis (V=6.25 L; CL=0.16 L/h) were weight-corrected (V=1.80 L/kg and CL=0.046 L/h/kg). In the individual analysis, the AUC calculated for the entire study period

was $17.44 \pm 9.92 \mu\text{g/mL} \cdot \text{h}$, whereas in 24 h the AUC was $4.35 \mu\text{g/mL} \cdot \text{h}$. The high variability obtained in most parameters was mainly due to the high values of patient M001.

The fitting of the data obtained with the model described in the methods section and the relative estimates of the pharmacokinetic parameters are reported in Figure 3. These estimates are in agreement with the mean values calculated for the individual patients (Table 3).

3.4 / Simulation of melatonin pharmacokinetics following repeated melatonin infusions

We used the *in vivo* data obtained in the PK study in neonates to estimate *in silico* the PK profile of repeated melatonin infusions. This approach allowed us to assess the number of infusions required to reach the steady-state and the AUC values. The simulation was based on the hypothesis that melatonin followed the same PK of the first infusion for each subsequent administration. Figure 4 shows that the steady-state can be reached after 4 infusions repeated every 24 h. According to the model, the lowest and highest concentrations that can be reached with this dosage are $0.31 \mu\text{g/mL}$ and $0.48 \mu\text{g/mL}$, respectively. The expected AUC at the steady-state after the 4th virtual infusion is $9.71 \mu\text{g/mL} \cdot \text{hr}$.

4 | DISCUSSION

When preclinical studies of a new medication are extrapolated to clinical studies, a dose that is effective and safe must be determined. This extrapolation is particularly difficult in neonatal studies because varied maturation rates and developmental ages can vary dose responses. Because the PK and safety profile of a new medication is unknown, a dose escalation study is the first investigative step. In a dose escalation study, a low dose is administered first to a group of patients. If safety issues do not arise, a new group of patients receives a higher dose.²²

The results reported here are the first data obtained in an FDA-approved clinical study that use the dose-escalation approach to identify a safe and potentially effective dose of melatonin for neonates with HIE undergoing hypothermia. In preclinical experiments the most effective melatonin dose was 10-15 mg/kg,^{4,11,23} although efficacy was also demonstrated at lower doses.^{11,14} When preclinical studies are translated into clinical experimentation, researchers must consider allometry when extrapolating the dosing.²⁴ Generally, in clinical studies performed in adult subjects, a linear correlation between dose and body surface area is assumed. Dose extrapolation is more complex in neonates because the drug PK may be different due to varied rates of maturation and development of the different organs.^{22,24,25}

Based on previous studies of the safety of melatonin administration,^{15,16} we started a dose escalation study to assess the safety and PK in neonates undergoing hypothermia (ClinicalTrials.gov Identifier: NCT02621944). We started with a melatonin dose of 0.5 mg/kg. This dose was chosen based on our previous study of melatonin administration in preterm neonates.²⁰ This previous study showed a higher half-life of melatonin in neonates as compared to animals and adults. Notably, Merchant et al. (2013) found similar results.²⁶ To our knowledge we are the first group to carry out a structured study that assesses the safety and PK profile of enterally-administered melatonin in neonates with a diagnosis of HIE and undergoing hypothermia.

4.1 | Melatonin tolerance

We administered 0.5 mg/kg melatonin enterally to neonates with HIE undergoing therapeutic hypothermia. The melatonin was well tolerated and did not result in any abdominal complications. Neither emesis nor abdominal distension occurred during or after the infusion. Serial abdominal exams during and after the infusion did not reveal any

changes. In addition, none of the neonates experienced increased somnolence or sedation during or after the melatonin infusion. The patients' vital signs did not exhibit major clinical changes during or after the infusion. The heart rates of patients M001, M002, and M003 decreased over time, which is consistent with neonates undergoing hypothermia who did not receive melatonin. The neonates in our study were treated based on an established hypothermia protocol that uses routine sedation with fentanyl. One known effect of fentanyl is a decreased heart rate. Patient M002's low heart rate during the infusion was most likely due to over sedation with fentanyl. This patient's heart rate was already decreasing to the 70-80 range in the hour prior to infusion. When the sedation was decreased during the infusion, the heart rate of patient M002 rose.

4.2 | Melatonin pharmacokinetics and dose extrapolation

We found that four of the five enrolled neonates showed low but detectable basal melatonin values (median value 20.6 pg/mL). These low basal melatonin values were similar to the values in our previous study of preterm neonates.²⁰ In this study, we enterally infused a new proprietary melatonin preparation that did not contain excipients. A preparation without excipients reduces the possibility of toxic effects by unwanted substances.

Our results show that the melatonin preparation was well absorbed enterally by neonates undergoing hypothermia. After infusion with 0.5 mg/kg melatonin, the C_{\max} reached 0.25 µg/ml. In an earlier study the same dose was administered as a single bolus to preterm neonates, who subsequently experienced a higher C_{\max} value (0.44 µg/ml).²⁰ The T_{\max} estimated in the population analysis was 6.80 h, and the individual patient T_{\max} mean was 8.66 h, range 6.5-13 h. The T_{\max} varied widely among patients.

In our population study in which melatonin was infused over time, the AUC was 4.35 $\mu\text{g/mL}\cdot\text{h}$ at 24 h. This AUC was lower compared to the AUC of 10.48 $\mu\text{g/mL}\cdot\text{h}$ found in a previous study that administered the same dose of melatonin as a single bolus.²⁰ The melatonin half-life and CL were prolonged, and V was decreased compared to adult²⁷ and animal models.²⁸ This finding is consistent with previous data obtained in preterm neonates.^{20,26} The inter-individual variations in the PK parameters in our study could be caused by differences in absorption, distribution, metabolism, or excretion, but the exact causes and clinical implications remain unestablished. Studies aimed at addressing these variability issue are in progress.

The *in silico* study simulated 7 infusions of melatonin repeated every 24 h. The simulation indicated that after each infusion, melatonin would follow the same PK of the first infusion. Using this approach, we estimated that the steady state would be reached after four infusions. This *in silico* analysis also showed that at the steady state the C_{max} and AUC values would be similar to those previously obtained after bolus administration (estimate after infusion: C_{max} 0.48 $\mu\text{g/mL}$, AUC 9.71 $\mu\text{g/mL}\cdot\text{h}$; bolus administration: C_{max} 0.44 $\mu\text{g/mL}$, AUC 10.48 $\mu\text{g/mL}\cdot\text{h}$).²⁰ Overall, these data, compared with our previous study in preterm neonates, indicate that hypothermia does not affect the PK of melatonin. In addition, high blood concentrations of melatonin in humans are achievable with lower doses compared to animal dosing.

Recently, we assessed the protective effect of melatonin in organotypic slice cultures of the hippocampus. Organotypic slice cultures maintain several aspects of structural and synaptic organization of the original tissue. In addition, the maturation of varied cell types and cooperation between neurons and glial cells are similar in organotypic slice cultures and the original tissue. Because organotypic slice cultures behave in similar ways to the original tissue, we were able to investigate the effects of melatonin on the hippocampus *in vitro* and

extrapolate the results to the *in vivo* situation.⁵ Using this system, we found a synergy between melatonin and hypothermia with melatonin concentrations of 25 μM and 50 μM and 6 hours of hypothermia. In the present study, we found that after a single infusion of 0.5 mg/kg melatonin, the AUC was 4.35 $\mu\text{g/mL}\cdot\text{h}$, equivalent to 18.73 μM . If we assume that our *in silico* simulation is correct, at the steady state the AUC should be 9.71 $\mu\text{g/mL}\cdot\text{h}$, equivalent to 41.51 μM . This steady state concentration is in line with the effective concentration found in our *in vitro* experiments.⁵

In our previous work we found a direct correlation between the dose of melatonin and the AUC.²⁰ The calculated AUC ratio of infusion/bolus of 0.5 mg/kg melatonin is 0.41 (4.35 $\mu\text{g/mL}\cdot\text{h}$ vs 10.48 $\mu\text{g/mL}\cdot\text{h}$, respectively). Thus, after an enteral infusion of 5 mg/kg melatonin, the estimated AUC could reach about 48 $\mu\text{g/mL}\cdot\text{h}$, equivalent to 207 μM . If melatonin is given intravenously, as in the case of Merchant et al.(2013),²⁶ a 0.5 mg/kg dose should also result in higher AUC values and faster steady-state than an enterally administered dose. If we assume that these extrapolations are correct, then the concentration obtained with these low doses should be even higher than the doses that showed synergy with hypothermia in studies with hippocampal slices.⁵ Currently we are treating patients with melatonin doses up to 5 mg/kg to assess safety and confirm our PK estimates.

4.3 | Conclusions

In the present study we administered melatonin by enteral infusion because it was the safest route for a study in neonates. The preparation is also suitable for intravenous infusion. The dose administered was the lowest dose proposed in the study approved by the FDA. The results of this study give important indications for future clinical studies aimed at assessing melatonin efficacy in HIE. The dose of melatonin, depending on the route of administration, should not exceed 1-5 mg/kg. This dosing range results in AUC values higher than effective

AUC values in hippocampal slices treated with hypothermia. At these doses, melatonin can be administered without the presence of excipients in the preparation, reducing the risk of side effects by unwanted substances. In addition, IV administration is preferred because bioavailability is 100%. IV administration of melatonin bypasses absorption through the gastric mucosa, so blood concentrations vary less among patients, and an effective blood concentration is achieved faster. We trust that our results will lay the foundation for larger randomized studies aimed at evaluating the efficacy of melatonin in association with hypothermia or when hypothermia cannot be applied.

Currently the length of melatonin treatment in HIE for optimal neuroprotection is unknown. Preclinical studies are necessary because new evidence shows that melatonin can enhance cell and tissue regeneration and is protective during acute neurodegeneration.^{29,30}

The injured immature brain holds high-level neural plasticity and could be particularly susceptible to treatment modalities such as melatonin that can trigger neuron generation (reactive neurogenesis) and creation of new neural networks.

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Authorship

Walter Balduini and Michael Weiss contributed to concept and design of the study, data acquisition, data analysis and interpretation, drafting and critical revision of the manuscript.

Walter Balduini contributed to pharmacokinetic studies and analysis.

Silvia Carloni contributed to pharmacokinetic studies, data analysis and interpretation, and manuscript review.

Marco Rocchi performed the pharmacokinetic studies, data analysis and contributed to data interpretation and manuscript review.

Livia Sura contributed to samples collection, data acquisition, and manuscript review.

Candace Rossignol contributed to samples collection and analysis.

Mariangela Longini contributed to data analysis and interpretation and manuscript review.

Francesco Bazzini contributed data analysis and interpretation and manuscript review.

Serafina Perrone contributed data analysis and interpretation and manuscript review.

Deborah Ott contributed to samples collection and data acquisition.

Rajan Wadhawan contributed to design of the study, data acquisition, and critical review of the manuscript.

Giuseppe Buonocore contributed to concept and design of the study, data acquisition, data analysis and interpretation, drafting and critical revision of the manuscript, and approval of the manuscript.

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- Accepted Article
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LEGEND TO FIGURES

Figure 1

The heart rate, mean arterial blood pressure (MAP), Systemic Saturations and Cerebral Saturations for subject M001 (Figure 1A, panels *a-d*), M002 (Figure 1B, panels *e-h*), M003 (Figure 1C, panels *i-n*), M004 (Figure 1D, panels *o-r*), M005 (Figure 1E, panels *s-v*). The parameters are shown prior to infusion (PI), during the infusion (DI) and after the infusion (AI) of melatonin (mean \pm SEM).

Figure 2

The abdominal circumference (cm) for subjects M001-M005 are graphed over time (hours). Time point 0 was prior to the infusion and represented a baseline measurement.

Figure 3

Population analysis of plasma melatonin concentrations after enteral infusion of 0.5 mg/kg melatonin in neonates subjected to hypothermia. A) Measured (symbols) and predicted (line) melatonin concentrations. B) Pharmacokinetic parameter estimates in the population analysis. The mathematical model used for this analysis is reported in the Methods section.

Figure 4

In silico pharmacokinetic profile of melatonin infusions repeated each 24 h. The simulation was based on the hypothesis that after each administration melatonin followed the same pharmacokinetic profile of the first infusion. The mathematical model used for this analysis is reported in the Methods section.

Table 1: Demographic and clinical data of patients with HIE enrolled

Patient	M001	M002	M003	M004	M005	Mean \pm SE
GA (wks)	39	41	40	40	39	39.8 \pm 0.75
Sex (M:F)	M	M	M	F	M	
BW (Kg)	3.46	3.54	3.57	3.46	3.26	3.46 \pm 0.05
Apgar 1'	1	1	1	1	1	1
Apgar 5'	1	2	6	5	5	3.8 \pm 1.94
Apgar 10'	1	3	6	N/A	7	4.25 \pm 2.39
Initial pH	6.89	7.21	7.06	6.99	7.03	7.04 \pm 0.10
BD	-19	-15	-12	-9	-17	-14.4 \pm 3.56
SS	II	III	II	II	II	
Seizures	No	No	No	No	Yes (clinical)	
ECS	Yes	Yes	No	No	Yes	
Intubation in DR	Yes	Yes	No	Yes	Yes	
Outborn/Inborn	Outborn	Inborn	Inborn	Outborn	Outborn	
Vasopressors	No	No	No	No	Yes	
Hour of life hypothermia initiated	4	0	3	3.5	5	3.1 \pm 1.69
Hour of life melatonin administered	8	14	6	9	9	9.2 \pm 2.64

GA = Gestational Age, BW = Birth Weight, BD = Initial Base Deficit, SS = Initial Sarnat

Score, ECS = Emergency C-section, DR = Delivery Room.

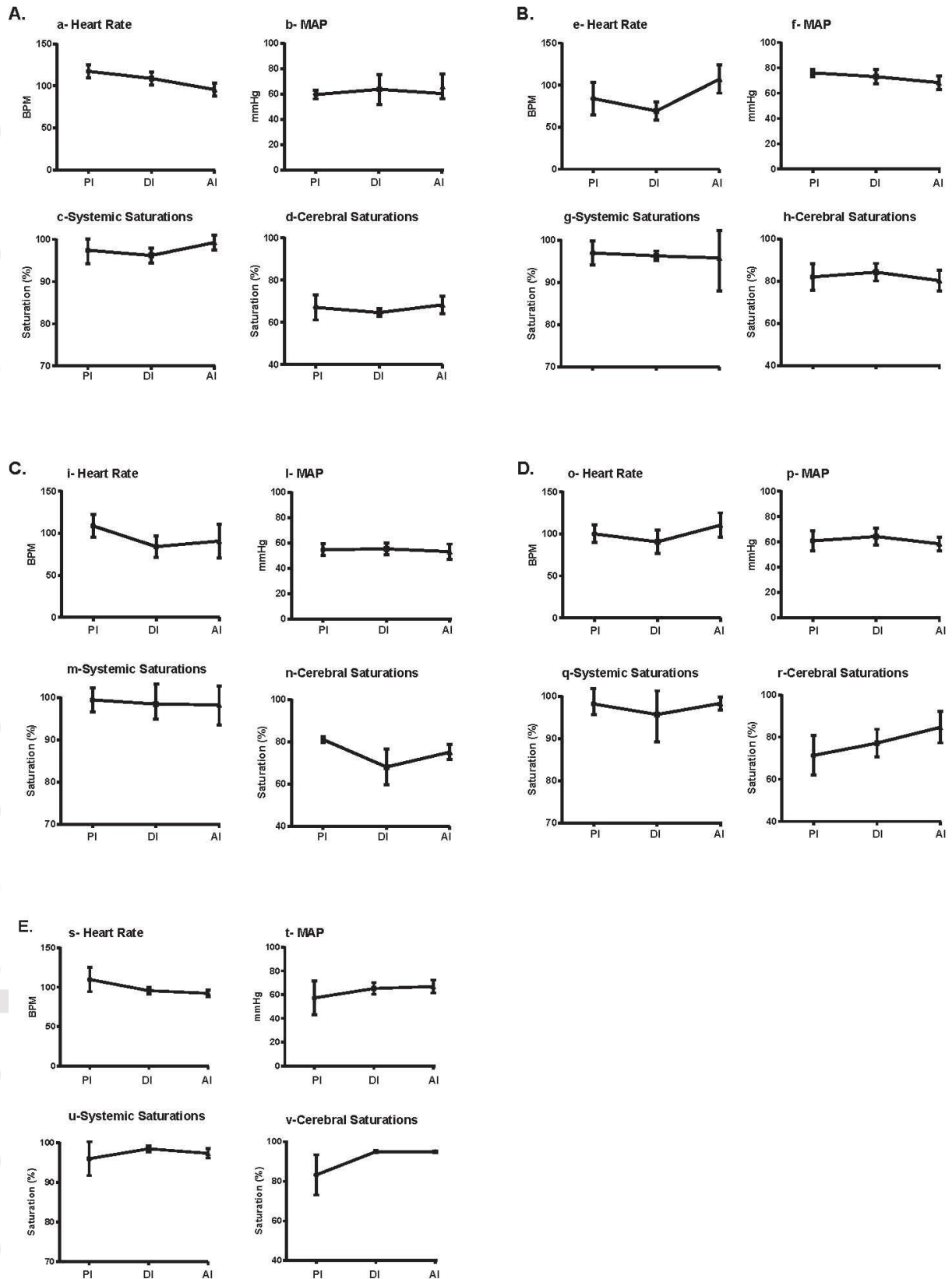
**Table 2: Melatonin plasma concentrations at different time after
0.5 mg/kg 4h-infusion**

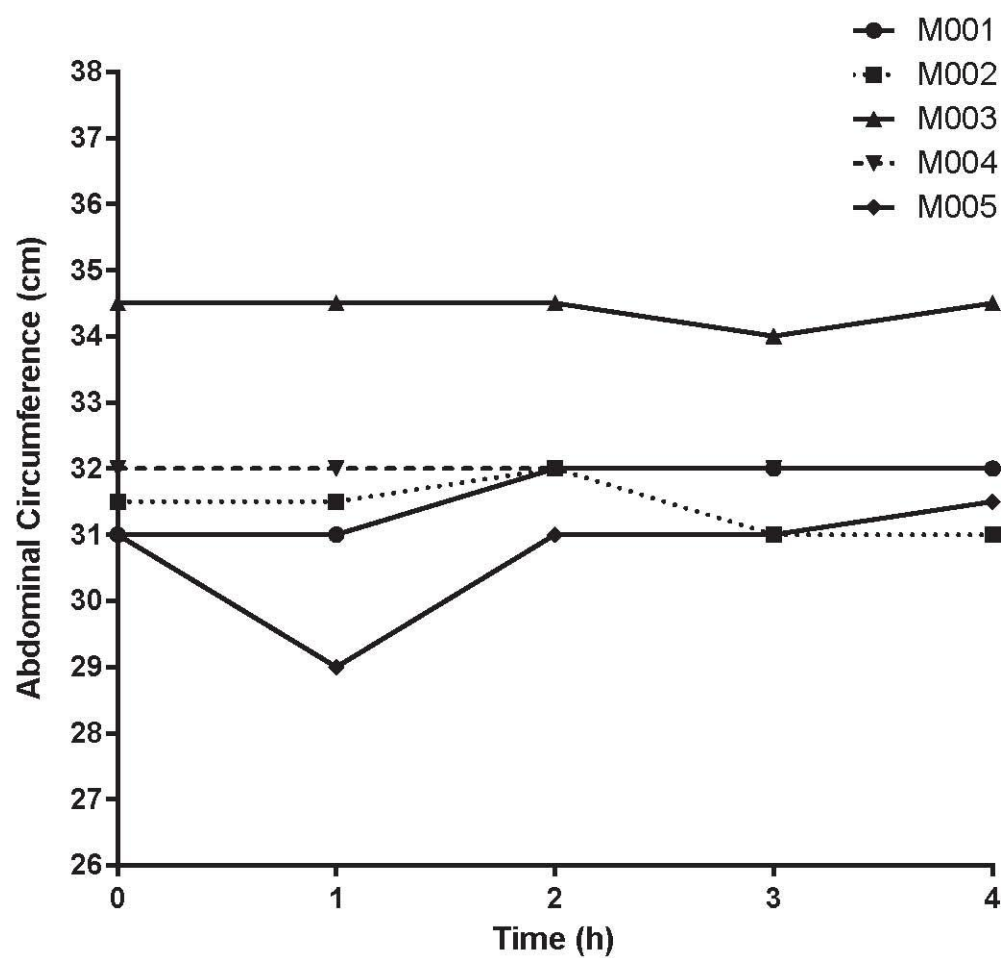
Melatonin 0.5 mg/kg 4h-infusion	Time (h)	Median (range) ($\mu\text{g/mL}$)
	0	20.6×10^{-6} (8.7×10^{-6} - 33.1×10^{-6})
	5	0.16 (0.13-0.34)
	7	0.20 (0.10-0.36)
	10	0.20 (0.17-0.37)
	16	0.24 (0.08-0.28)
	28	0.12 (0.04-0.23)
	52	0.04 (0.005-0.22)
	100	0.008 (0.001-0.10)

**Table 3. Estimates of melatonin pharmacokinetic parameters in patients with HIE
enrolled**

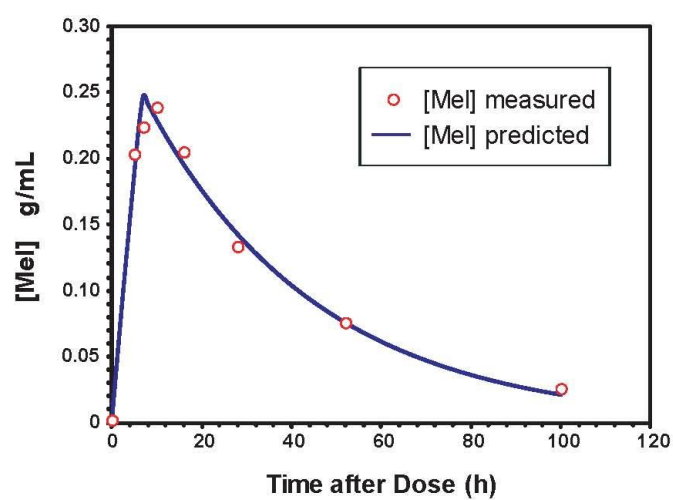
Patient	M001	M002	M003	M004	M005	Mean \pm SE
K_{el}	0.003	0.063	0.038	0.079	0.033	0.043 ± 0.012
$T_{1/2}$ (h)	196.33	10.97	17.83	8.74	20.57	50.90 ± 36.43
AUC ($\mu\text{g/mL/h}$)	56.92	8.76	8.68	3.64	9.20	17.44 ± 9.92
T_{max} (h)	7.8	7.9	13	6.5	8.1	8.66 ± 1.12
C_{max} ($\mu\text{g/mL}$)	0.19	0.43	0.26	0.22	0.27	0.27 ± 0.04
V(L/kg)	8.62	3.19	5.29	5.99	5.25	5.67 ± 0.08
CL (L/h)	0.03	0.20	0.20	0.47	0.17	0.21 ± 0.07

K_{el} , elimination constant; $T_{1/2}$, elimination half-time; AUC, Area Under concentration-time Curve; T_{max} , time to reach maximal serum concentration; C_{max} , maximal serum concentration; V, apparent Volume of distribution; CL, Clearance.





A



B

Parameter	Value
K_{el}	0.03
$T_{1/2}$ (h)	26.43
AUC ($\mu\text{g/mL}\cdot\text{h}$)	9.54
T_{max} (h)	6.8
C_{max} ($\mu\text{g/mL}$)	0.25
V (L/kg)	6.25
CL (L/h)	0.16

